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Protein and Enzyme Variation in Some Hevea Cultivars

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Serum proteins from latices of several Hevea *cultivars were separated electrophoretically using polyacrylamide gels. Analysis of the zymograms of a number of enzymes indicated that* H. brasiliensis, H. benthamiana *and their hybrid FX 2784 could be distinguished from one another. Some clonal differences were seen in the electropherograms of B-serum and of sera prepared by acetic acid coagulation of latex separated electrophoretically at acid pH.*

Since the discovery of molecular heterogeneity in lactate dehydrogenase in 1957[1,2] there has been increasing appreciation of the fact that enzymes commonly exist in a variety of molecular forms. Though in many cases their functional significance is not very clear, they have served as useful markers in a variety of physiological and genetic studies, and various aspects of isoenzyme research have been reviewed in recent years[3,4,5].

Multiple forms of several enzymes have been found in *Hevea* latex[6,7], but there appears to be no report of variations in these isoenzymes between cultivars of *Hevea*, and thus of the potential use of isoenzymes as markers in this crop. The recent studies of Walujono and Suseno[8], however, have indicated differences in electropherograms between clones of *Hevea*. The present study is an attempt to evaluate the use of polyacrylamide gel electropherograms and zymograms in identification of *Hevea* cultivars.

MATERIALS AND METHODS

Latex was collected from *Hevea benthamiana,* from various primary clones of *Hevea brasiliensis* and from FX 2784 (a hybrid between *H. brasiliensis* and *H. benthamiana*) growing in the RRIM Experiment Station at Sungei Buloh, and in regular S/2.d/2 tapping. The trees available for this study were in tapping on different panels, so it was not generally possible to compare latices collected from similar panels; but where cultivar differences were seen, collections from different fields were compared whenever possible to evaluate the possible effects of environment.

Latices were collected from individual trees and not pooled, except for collections from *H. benthamiana* where the yield from individual trees was poor. Latex was collected in ice-chilled containers and centrifuged at 26 000 r.p.m. (61 000 g max) for 1 h at a temperature between 3°C and 4°C using Rotor 40 of the Spinco Model L Ultracentrifuge. This resulted in the separation of latex into a rubber phase, a sediment (bottom fraction) and a liquid phase (C-serum) as described in the literature[9,10]. The cellulose nitrate tubes were subsequently punctured to drain out the C-serum which was then filtered through glass wool and stored at 5°C under refrigeration. To prepare B-serum, the bottom fraction was washed twice by suspending in 0.4M mannitol, and then disrupted by freezing and thawing[11] in a mixture of alcohol and dry-ice. B-serum from the ruptured bottom fraction vesicles was recovered as the suspernatent after centrifugation at 20 000 r.p.m. (49 000 g max) in a Sorvall RC 2B centrifuge at 4°C for 30 minutes. The serum was normally filtered through Whatman's No. 1 filter paper

COMMUNICATION 603

before use. A serum was also prepared by coagulation of whole latex with 2% acetic acid at 7°C (2 vol latex: 1 vol acid), following Walujono and Suseno[8]. All serum samples were normally used without dialysis within 6 h of preparation.

Protein concentration in the serum samples were determined by preciptating the protein with 5% trichloroacetic acid, redissolving in 0.25% sodium hydroxide, and assaying by the Lowry reaction[12].

To release vesicle bound B-serum into whole latex prior to centrifugation latices were incubated with 0.125% Triton X-100 at 37°C for 30 min (1 vol latex: 4 vol Triton X-100)[13].

Both acidic and basic proteins are found in C-serum as well as in B-serum[6]. Proteins of C-serum were electrophoretically separated at both alkaline and acid pH, while proteins in B-serum and from acid-coagulated latex were separated in acid buffers only. For separation of proteins at alkaline pH, the cathode was applied to the origin while for separation of proteins in acid pH the anode was applied at the origin.

Electrophoresis at *alkaline pH* was carried out according to Ornstein and Davies[14,15], using Tris-glycine buffer at pH 8.3. The samples were applied in 0.2 ml aliquots containing serum diluted to about 250 μg protein. Bromophenol blue was used as tracker and a current of 3 mA per tube was applied.

Electrophoresis at *acid pH* was carried out according to Reisfeld *et al.*[16] using β-alanine-acetate buffer at pH 4 and 4.5. The samples were applied in 0.1 ml to 0.4 ml aliquots and contained about 250 μg to 700 μg protein. Generally, higher concentrations of protein were required for separating C-serum, while much lower concentrations were used for B-serum. Methyl green was used as tracker and a current of 6 mA per tube was applied.

Polycrylamide gels were prepared in 10cm perspex tubes with an internal diameter of 6 mm. Samples were weighted by sucrose to a final concentration of 40% (w/v). Electrophoresis was carried out for about 2 h in a refrigerator using a Shandon apparatus.

Gels were stained for protein either in 0.15% Naphthalene Black prepared in 7% acetic acid or in 0.05% Coomassie Blue according to Chrambach *et al.*[17].

The procedures outlined by Lee and Dougall[18] were followed for locating isoenzymes of malic dehydrogenase (EC 1.1.1.37), aspartate amino transferase (EC 2.6.1.1) and esterase (EC 3.1.1.1). Dihydroxyphenylalanine (DOPA) was used to locate polyphenol oxidase (EC 1.14.18.1) according to Nye *et al.*[19]. The methods outlined by Sakai *et al.*[20] were followed for peroxidase (EC 1.11.1.7) except that the concentration of H_2O_2 was increased ten times. Acid phosphatase (EC 3.1.3.2) was located following Work and Work[21]. Generally in staining for isoenzymes, where the gel pH differed considerably from the pH for enzyme reaction, the gels were first equilibrated for 30 min in the same buffer used for the enzyme reaction.

RESULTS AND DISCUSSION

Clonal Differences in Protein Electropherograms

Up to twenty-six protein bands were observed in C-serum after electrophoresis at *alkaline pH (Figure 1).* However, no consistent differences were discernible between electropherograms of five clones of *H. brasiliensis, viz:* Tjir 1, PR 107, GT 1, PB 86 and BR 2.

Electrophoresis of C-serum at *acid pH* revealed about fifteen bands, most of them faint *(Figure 2)*; but similarly, no consistent differences between Tjir 1, PR 107 and GT 1 were observed.

Inconsistencies in the electrophoretic patterns appeared in part to be due to the varying degree of contamination of C-serum by B-serum released from the bottom

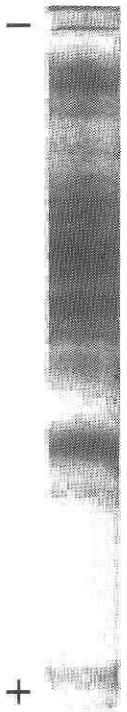

Figure 1. Electropherogram of Tjir 1 C-serum proteins separated at alkaline pH. Stained with Naphthalene Black.

fraction. As will be discussed below, B-serum contamination could be expected to be particularly significant in acid pH runs.

Serum samples from Tjir 1, PR 107, GT 1, LCB 1320 and WR 101 were prepared by acetic acid coagulation of latex, following the procedure of Walujono and Suseno[8]. As reported by Walujono and Suseno, some consistent differences were discernible following electrophoresis at acid pH *(Figure 3).* Most of these occurred in the fast migrating, deep staining bands. The electropherogram of Tjir 1 in particular was distinguishable from all the others. Clones PR 107 and GT 1 had somewhat similar patterns and could not be readily distinguished from each other. Similarly, it was not possible to distinguish between the electropherograms of WR 101 and LCB 1320.

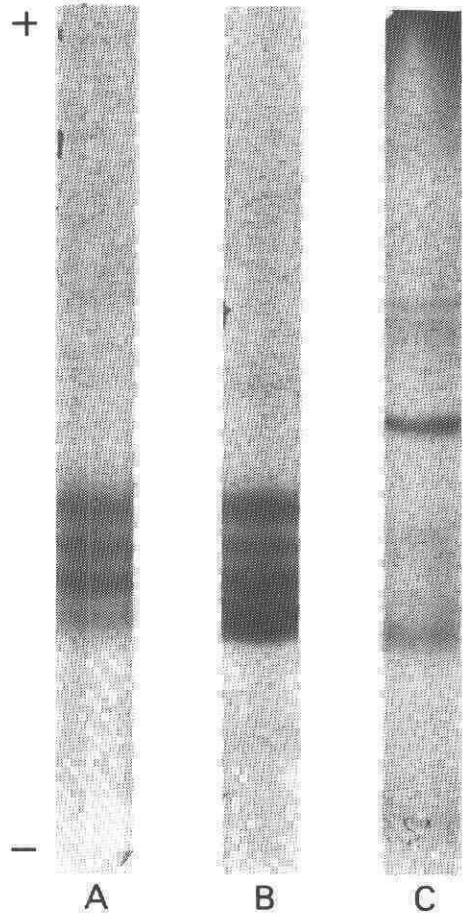

Figure 2. Electrophoresis at acid pH of GT 1 serum prepared by acetic acid coagulation of latex (A), compared with B-serum (B) and C-serum (C) of the same clone. The fast migrating, deeply stained bands are prominent in A and B. Stained with Coomassie Blue.

Electropherograms of serum prepared by acid coagulation of latex (presumably a mixture of B- and C-sera) contained a few bands corresponding to those in electropherograms of C-serum prepared by ultracentrifugation. The most prominent bands in electropherograms of serum from coagulated latex (where clonal differences were discernible, as described above) were never-

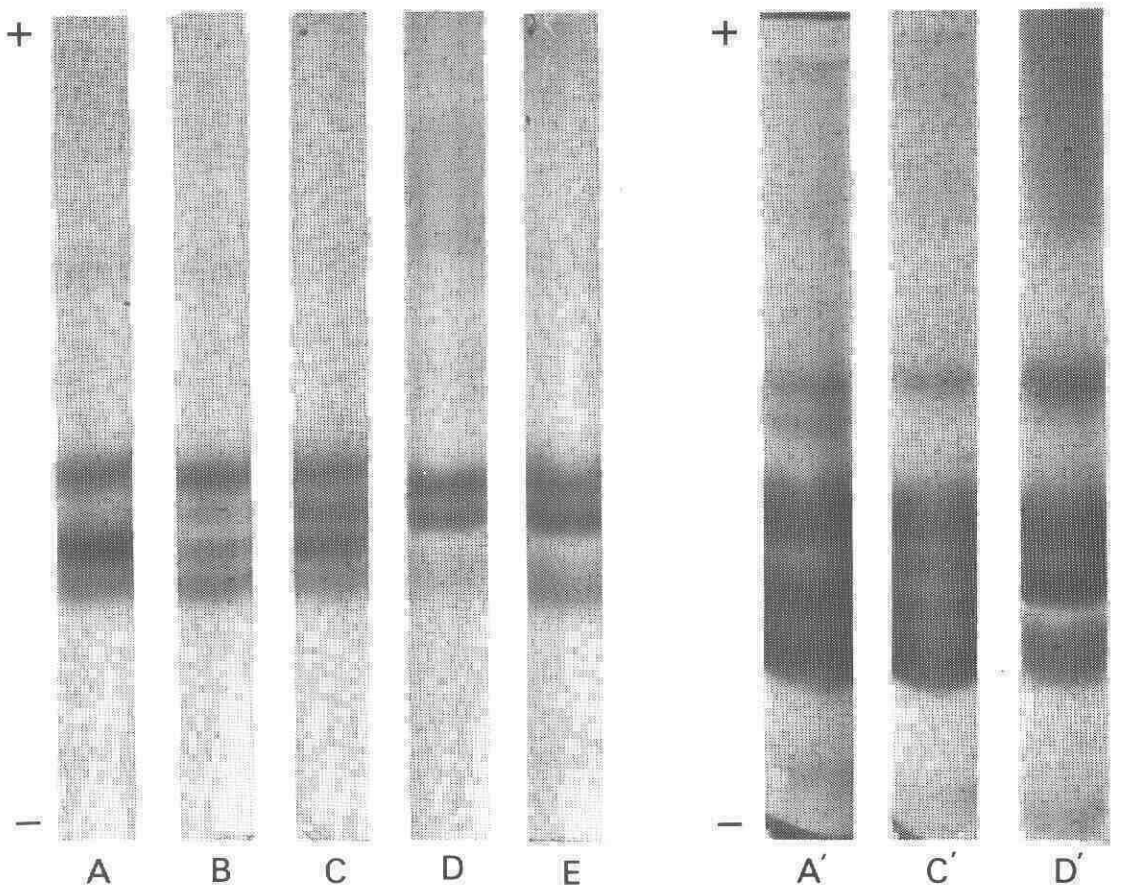

Figure 3. Electrophoresis at acid pH of serum prepared by acetic acid coagulation of latex (A−E), and of B-serum (A′−D′). Clonal differences are discernible in the fast migrating bands. Stained with Coomassie Blue.

A: Tjir 1 serum from acetic acid coagulation of latex.
A′: Tjir 1 B-serum.
B: PR 107 serum from acetic acid coagulation of latex.
C: GT 1 serum from acetic acid coagulation of latex.
C′: GT 1 B-serum.
D: WR 101 serum from acetic acid coagulation of latex.
D′: WR 101 B-serum.
E: LCB 1320 serum from acetic acid coagulation of latex.

theless faint or absent in electropherograms of serum from centrifuged latex. These bands were, however, clearly evident in B-serum electropherograms of the same clone *(Figure 2)*. Furthermore, serum prepared by ultracentrifugation of latex pre-treated with Triton X-100 (which ruptures lutoids and other vesicles) gave patterns very similar to those of serum from acid-coagulated latex *(Figure 4)*. It thus appeared that a large number of the cationic protein bands obtained by electrophoresis of serum from acid-coagulated latex were derived from B-serum proteins. To confirm this, B-serum, C-serum and 2% acetic acid were mixed in a ratio such as would normally occur in acid

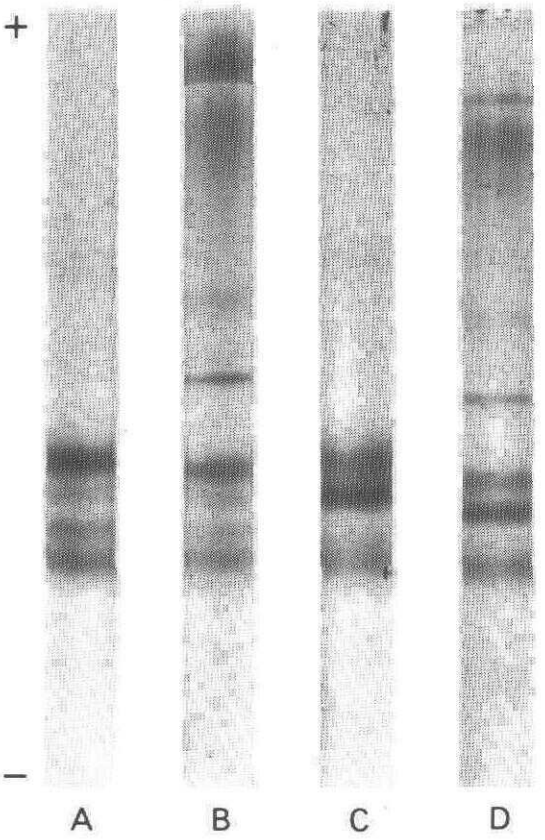

Figure 4. Electrophoresis at acid pH of serum prepared by acetic acid coagulation of latex and of serum prepared from Triton X-100-treated latex.

A: PR 107 serum from acetic acid coagulation of latex.

B: PR 107 serum from Triton X-100-treated latex.

C: WR 101 serum from acetic acid coagulation of latex.

D: WR 101 serum from Triton X-100-treated latex.

Similarities in the electropherograms of serum samples prepared by the two methods are evident. Stained with Coomassie Blue.

coagulation of latex, *viz.* 10 vol acid: 5 vol C-serum: 2 vol B-serum. (For this study, the volumes of C-serum and B-serum obtained by centrifugation of latex were found to be approximately 25% and 10% respectively of whole latex, and this was used as a basis in the above formulation.) In control samples, the acetic acid component of the mixture was replaced by water to determine if acetic acid treatment resulted in significant loss of protein bands by precipitation. In all instances, any precipitate that appeared was discarded. Upon electrophoresis, B-serum protein bands (especially those of the fast migrating zone where clonal differences were detected) were found to be predominant in mixtures containing B-serum, irrespective of the presence or absence of acetic acid *(Figure 5)*. This was despite the fact that C-serum was present in a greater proportion. As expected, when B-serum proteins of different clones were compared, clonal differences similar to those appearing in serum from acid-coagulated latex were seen *(Figure 3)*.

Cultivar Differences in Zymograms

Following electrophoresis at *alkaline pH*, isoenzymes of esterase, peroxidase and polyphenol oxidase from C-serum *(Figure 6)* of *H. benthamiana* and the *benthamiana-brasiliensis* hybrid FX 2784 could be readily distinguished from each other and from the clones of *H. brasiliensis*. No consistent differences were found in isoenzyme patterns between the *H. brasiliensis* clones Tjir 1, PR 107 and GT 1. The presence of the major bands was consistent, but the intensity and presence or absence of some of the minor bands of a clone varied from sample to sample. This was particularly evident in polyphenol oxidase, peroxidase, esterase and acid phosphatase zymograms. It may be significant that the latter enzymes are to be found in greater quantities in the B-serum[22,23] of latex, the variation therefore possibly arising from day to day differences in damage to latex organelles (lutoids and Frey-

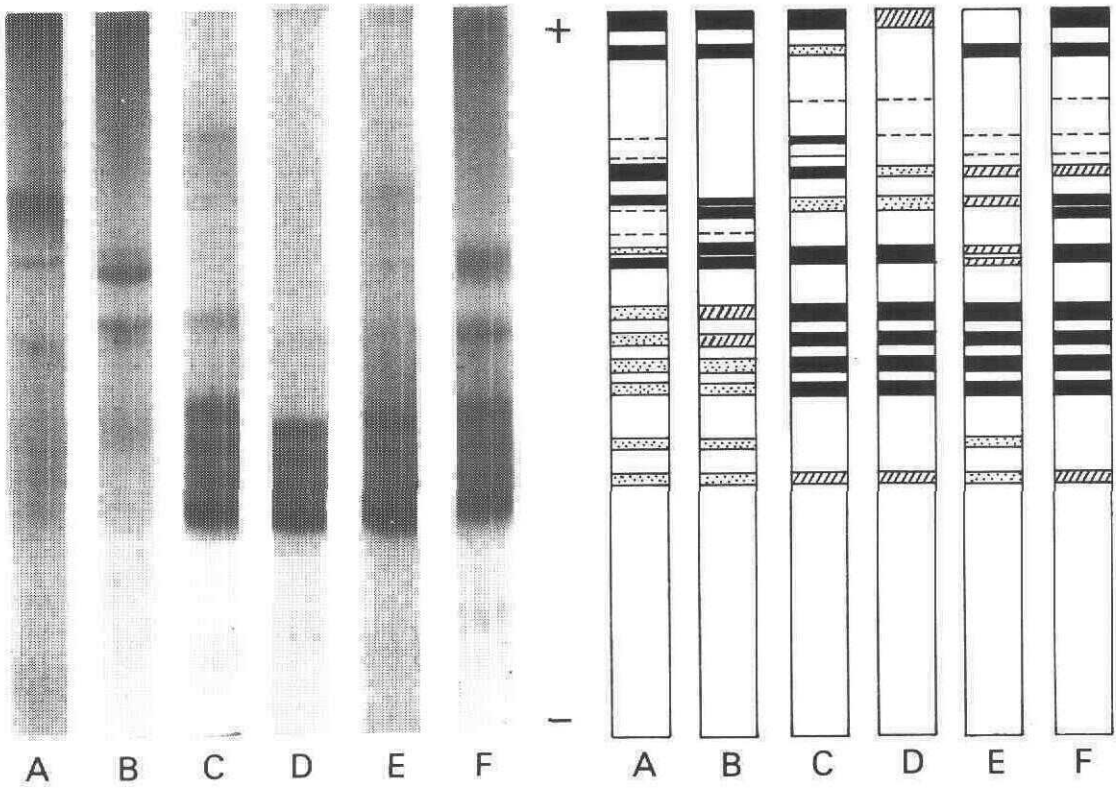

Figure 5. PR107 B-serum, C-serum and mixtures of these run at acid pH (diagramatic representations are given on the right).
A: 10 ml 2% acetic acid + 2 ml water + 5 ml C-serum.
B: 12 ml water + 5 ml C-serum.
C: 10 ml 2% acetic acid + 5 ml water + 2 ml B-serum.
D: 15 ml water + 2 ml B-serum.
E: 10 ml 2% acetic acid + 5 ml C-serum + 2 ml B-serum.
F: 10 ml water + 5 ml C-serum + 2 ml B-serum
Stained with Coomassic Blue.

Wyssling complexes) or in leakage of the enzymes from them.

Differences in the zymograms were not always seen in the presence or absence of particular bands, but often in the degree of enzyme activity as revealed by the intensity of staining. It was observed that some isoenzymes in sera from *H. benthamiana* showed a stronger staining reaction than *H. brasiliensis (e.g.* esterase, peroxidase, polyphenol oxidase), and that the hybrid FX 2784 was intermediate in this respect. In the isoenzymes of aspartate amino transferase (an enzyme hitherto unreported in *Hevea*), the faster migrating zone of bands was of interest as three distinct bands appeared in the hybrid FX 2784 whereas only two were seen in *H. brasiliensis* and *H. benthamiana* cultivars (*Figure 6e*).

When electrophoresis was carried out at *acid pH*, isoenzymes of esterase, peroxidase, polyphenol oxidase and acid phosphatase of

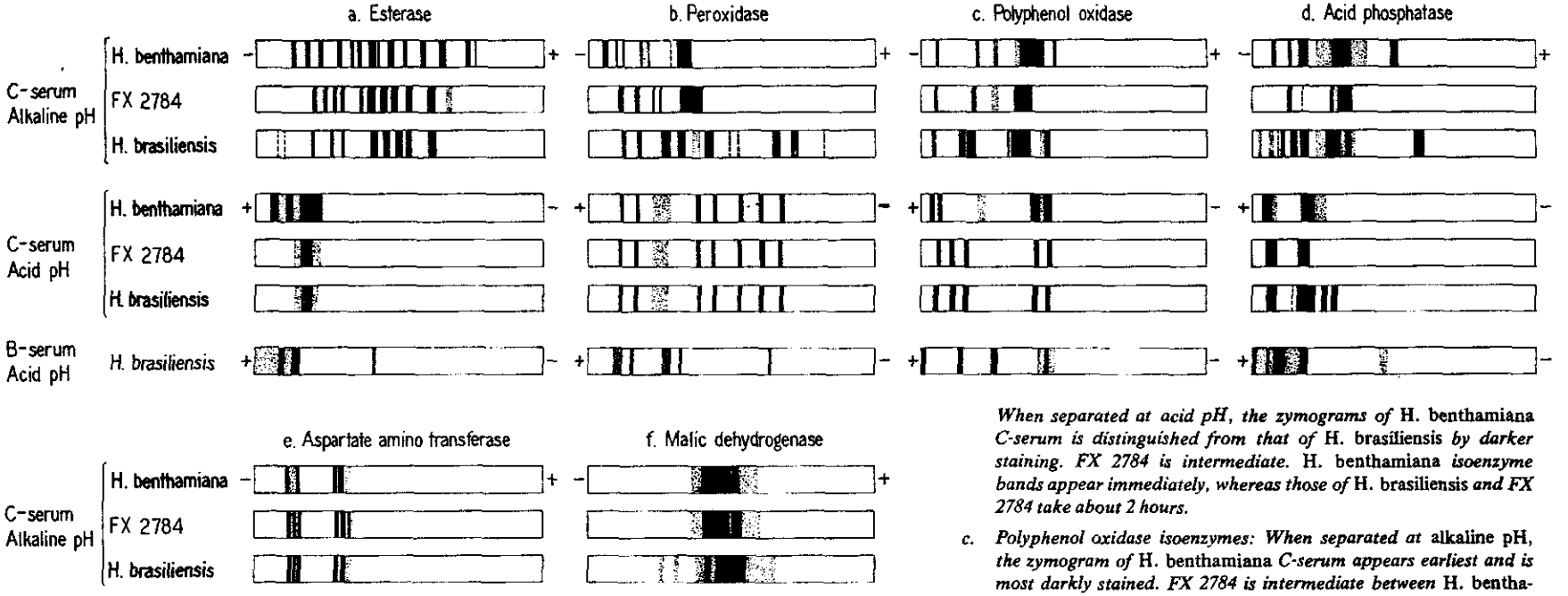

Figure 6. C-serum isoenzymes following electrophoresis at alkaline pH, and B-serum isoenzymes following electrophoresis at acid pH (diagramatic representations).

a. *Esterase isoenzymes: When separated at alkaline pH, the zymogram of* H. benthamiana *C-serum is distinguished from that of* H. brasiliensis *by a larger number of bands, and by an earlier appearance of bands. FX 2784 is intermediate in both these respects.*

   When separated at acid pH, H. benthamiana *C-serum isoenzymes are stained darkest.*

b. *Peroxidase isoenzymes: When separated at alkaline pH, the zymogram of* H. benthamiana *C-serum is distinguished from that of* H. brasiliensis *by an earlier appearance of the bands and by darker staining. FX 2784 is intermediate in both these respects.*

   When separated at acid pH, the zymograms of H. benthamiana *C-serum is distinguished from that of* H. brasiliensis *by darker staining. FX 2784 is intermediate.* H. benthamiana *isoenzyme bands appear immediately, whereas those of* H. brasiliensis *and FX 2784 take about 2 hours.*

c. *Polyphenol oxidase isoenzymes: When separated at* alkaline pH, *the zymogram of* H. benthamiana *C-serum appears earliest and is most darkly stained. FX 2784 is intermediate between* H. benthamiana *and* H. brasiliensis.

d. *Acid phosphatase isoenzymes: In most instances, C-serum zymograms vary with different latex collections.*

e. *Aspartate amino transferase isoenzymes: When separated at alkaline pH, the zymogram of FX 2784 C-serum is distinguished by the division of the fast migrating activity zone into three distinct bands.*

   No bands were detected in C-serum separated at acid pH.

f. *Malic dehydrogenase isoenzymes: The main activity zone usually appears as an undifferentiated large band for all three cultivars, when C-serum is separated at alkaline pH.*

   No bands were detected in C-serum separated at acid pH.

B- and C-sera of Tjir 1, PR 107 and GT 1 showed no consistent clonal differences in their zymograms *(Figure 6)*. Some isoenzyme bands in B-serum showed the same electrophoretic mobility as bands in C-serum. This could have been due to leakage of B-serum enzymes from damaged organelles *(e.g.* acid phosphatase from lutoids) into the C-serum.

This study shows that a degree of clonal identification is possible by electrophoresis at acid pH of B-serum or serum from latex coagulation. However, further ambiguities might be expected when a wider range of clones is studied.

Isoenzyme analysis was effective in distinguishing between the two *Hevea* species and their hybrid although it has not as yet proved adequate in distinguishing cultivars at the clonal level. A study of the numerous other enzymes that are present in *Hevea* latex might prove more conclusive in demonstrating clonal differences. In particular, a study of the enzymes associated with the fast migrating protein bands where clonal differences have been observed might prove useful.

Nevertheless, protein in latex sera can be electrophoretically separated with minimal pre-treatment, and it can be expected that this could be a useful tool in areas where a greater variation might be expected, as in studies on gene pool variations in natural seedling populations of *Hevea.* It is also plausible that a study of several clones might reveal correlations between isoenzymes patterns and desirable traits such as disease resistance or high yield.

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